A RAPID AND EFFICIENT METHOD OF PRODUCING HEMOLYTIC AMBOCEPTOR AGAINST SHEEP CORPUSCLES *

ARTHUR F. COCA

From the Department of Experimental Pathology in the Medical College of Cornell University, New York City

From the time when the principle of complement-deviation was applied to the serum diagnosis of disease, as in syphilis, gonococcic infection, glanders, and malignant tumor, the possession of a "good" hemolytic immune-serum, usually prepared against sheep corpuscles, became a necessity to the clinical laboratory. By a "good" hemolytic serum is meant one that possesses a sufficiently high hemolytic strength, which must remain permanently constant.

The usual method of producing hemolytic sera has been to inject relatively large and increasing quantities (5-20 c.c., in some laboratories even as much as 50 or 75 c.c.) of the washed corpuscles at intervals varying from three days to one week, until four or five injections have been given, the bleeding of the animals taking place about one week after the last injection. The results obtained by this procedure have been often unsatisfactory, it being almost exceptional for the hemolytic serum thus produced to possess both of the requirements mentioned.

Following the publication by Fornet and Muller[1] of experiments showing that precipitating sera of high potency can be produced by injecting the antigen in increasing amounts on three successive days, this rapid method of immunization was applied by Bonhoff and Tsuzuki,[2] without success, to the production of hemolytic sera against the corpuscles of the sheep, the horse, man, and the pig. The titer of the resulting sera was uniformly low, 1 : 10.

More recently Gay and Fitzgerald[3] repeated these experiments of Bonhoff and Tsuzuki, using only sheep blood corpuscles and injecting quantities of 1 or 2 c.c. intravenously. According to Gay and Fitzgerald, such injections, administered on three successive days, were fol-

* Received for publication June 8, 1915.

1. Ztschr. f. biol. Technik u. Methodik., 1908, 1, p. 201.
2. Ztschr. f. Immunitätsforschung, 1910, 4, p. 180.
3. University of California Publications in Pathology, 1912, 2, p. 79.

lowed by the development of an unusually strong hemolytic property in each of the eight animals used for the experiment.

For a number of years, the writer has studied the production of hemolytic sera in rabbits and has been able, on the basis of many observations, to formulate some generalizations that are of practical value to routine serology.

1. The first of these generalizations is the well-known fact that the individual rabbits differ greatly in their response to the injection of the foreign corpuscles. These individual differences, which are both quantitative and qualitative, are illustrated in the following experiment:

Six normal rabbits received, on June 16, 2 c.c. each of washed sheep corpuscles, intravenously injected, and on June 21 a second intraperitoneal injection of 10 c.c. each. On June 30, the sera of the rabbits were examined as to their hemolytic activity. In these tests, as in all the subsequent tests of hemolytic strength referred to in this communication, the different reagents were combined in one-tenth of the usual quantities, i. e., the different hemolytic sera were first diluted 1:10 and then mixed in descending amounts with 0.1 c.c. of a 5 percent suspension of washed sheep corpuscles (based on the volume of full blood taken and representing about 2.5 percent of corpuscular sediment) and 0.1 c.c. of a 1:10 dilution of fresh guinea-pig serum. The smallest quantity of the diluted hemolytic serum that caused complete hemolysis of the sheep corpuscles was noted as indicating the relative hemolytic strength of the serum.

The results of the test were:

In Rabbits 11, 12, 15, and 16	0.0002
In Rabbit 10	0.0004
In Rabbit 9	0.0008

Five weeks later, the sera that had been obtained from Rabbits 11, 12, 15, and 16 on June 30 and preserved by the addition of 0.25 percent of carbolic acid, were tested again. The results of this test with Sera 11, 15, and 16 were identical with those obtained at the first examination; whereas the titer of Serum 12 had fallen to about 0.0008, i. e., to about one-fourth of its original strength.

It is seen that the same previous treatment in the six rabbits resulted in a high hemolytic potency in only four of the animals, and that, of these four, only three furnished hemolysins that were entirely stable.

2. It has been found that the quality of the hemolysins produced by means of many injections is often different (as judged by the criteria of stability and anti-complementary property) from those obtained after few injections.

This qualitative difference was clearly seen when the immunization of Rabbits 11, 15, and 16 was resumed, as follows:

From August 9 until August 16, inclusive, daily intravenous injections of 0.2 c.c. of washed sheep corpuscles; August 25, 27, and 29 similar injections; September 23, 25, and 26 similar injections of 1.0 c.c. On October 2 — six days after the last injection — the sera of the three animals were examined; they were all found to possess the same hemolytic potency, namely, 0.0001. It was observed that when the larger quantities of the hemolytic sera (0.002 c.c. or more) were combined with the unit of sheep corpuscles, if complement were immediately added, hemolysis failed to occur; whereas, if the addition of complement was deferred for about fifteen minutes, solution of the corpuscles then followed. This phenomenon has never been noticed in testing hemolytic sera that had been obtained after few injections of the corpuscles. One-quarter of 1 percent of carbolic acid was mixed with the sera and after three weeks, during which the sera had been kept in the ice-box, they were again examined; it was found that all three of them had lost over 90 percent of their original strength. The titers were: Serum 11, 0.0015, Serum 15, 0.0014, and Serum 16, 0.0012. There was no bacterial growth in any of the sera. After another interval of one month, Sera 15 and 16 were found to have exactly the same hemolytic power as at the last examination. Serum 11 had been lost by accident.

These experiences, confirmed in a number of other rabbits that had had similar treatment, show that after many injections made over a relatively long period of time the resulting hemolytic property of the serum is often in large part—over 90 percent—unstable; whereas after few injections—two in the present instance—the same animals usually yield sera the hemolytic potency of which remains entirely constant; they show, furthermore, that hemolytic sera obtained after many injections of the corpuscles possess certain anticomplementary properties, which are not found in such sera obtained after few injections.

3. It was found that the maximal degree of immunity can be effected by injecting relatively small quantities of the blood corpuscles.

The most powerful hemolytic sera that we have examined were prepared by giving daily intravenous injections of as little as 0.1 c.c. of washed sheep corpuscles for a period of many weeks. Such sera have been hemolytic in quantities of 0.00005 and 0.000025 c.c. A hemolytic serum of equally high strength has been obtained by Dr. L'Esperance with the same procedure.

We have already demonstrated (under 2) that the important quality of stability of hemolytic strength combined with high pontency can be secured only by the administration of few injections, and, under this condition, as the following experiment shows, the maximal effect

cannot be produced with so small an amount as 0.1 c.c.* One cubic centimeter, however, was found to be sufficient.

Six normal rabbits that had received two intravenous injections of 0.1 c.c. of washed sheep corpuscles at intervals of four to six days yielded hemolytic sera of an average strength of 0.001. Four other normal rabbits that had received two similar injections of 1.0 c.c. each at an interval of five days yielded hemolytic sera of an average strength four and one-half times as great—0.00022. Nine additional normal rabbits receiving primary injections of 1 or 2 c.c. and second injections of 5 or 10 c.c. yielded sera possessing an average potency of 0.00025.

From the results of this experiment it is seen that no greater hemolytic activity is obtained by injecting 5 or 10 c.c. than by injecting 1 c.c. of the corpuscles.

4. It has been found that the optimal time for making the second injection of the sheep corpuscles is not earlier than at the end of an interval of three days after the first injection; that is, on the fourth day of the treatment.

The considerable advantage obtained by making the second injection after an interval of not less than three days is graphically shown in the accompanying table. The height of the black columns represents the average relative concentration of the hemolytic substances in the blood after the different immunizing procedures.

It is seen that the low average hemolytic power resulting from a single injection is considerably increased by further injections undertaken on the second and third days—Columns 2 and 3. This increase, however, is much greater if the second injection is deferred till the fifth day—Column 4.

5. The height of the hemolysin production following the first injection of sheep corpuscles is reached after an interval of seven days, and following the second injection the new height is reached after a further interval of not more than five days.

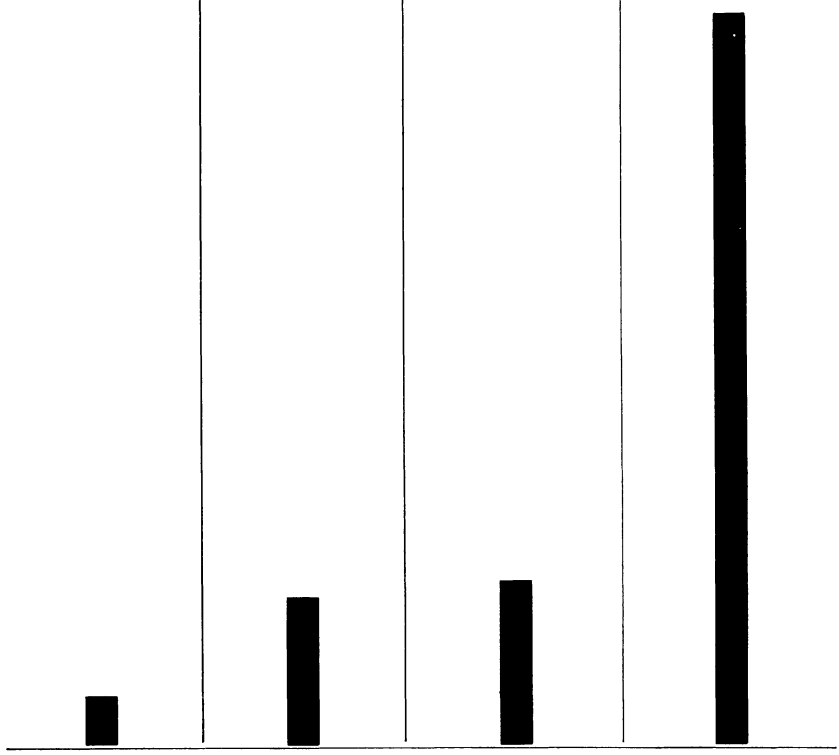
In the accompanying chart is shown the course of the hemolysin production in six rabbits that received two intravenous injections each

* This statement applies only when more than a single injection are given, as the following experience shows: Ten normal rabbits received 0.1 c.c. each of washed sheep blood corpuscles, intravenously administered, and one week later these animals yielded hemolytic sera of an average strength of 0.004. Three other normal rabbits that had received similar injections of 2.0 c.c., that is, twenty times as much as the former series, yielded an average hemolytic power of only 0.0065. This average would, no doubt, have been higher if a larger number of animals had been used; however, it is safe to assume that even then the average hemolytic power would not have been greater than that obtained in the series in which 0.1 c.c. of the corpuscles was injected.

TABLE 1

THE ADVANTAGE OF A FOUR-DAY INTERVAL BETWEEN THE INJECTIONS OVER A TWO-DAY INTERVAL AND THE SO-CALLED "RAPID METHOD" — COLUMN 3 — IN THE IMMUNIZATION OF RABBITS AGAINST SHEEP CORPUSCLES

AVERAGE TITER OF			
Thirteen Rabbits Receiving a Single Injection	Three Rabbits Receiving Two Injections, the Second Injection being Given on the Third Day	Four Rabbits Receiving Injections on Three Successive Days	Four Rabbits Receiving Two Injections, the Second Injection being Given on the Fifth Day
0.005	0.0012	0.001	0.00022



of 0.1 c.c. of washed sheep corpuscles at intervals of five, six, and seven days.

Following the first injection, the hemolysin content of the blood began to rise in all the animals after an interval of four or five days and increased daily up to the seventh day. Then, with one exception —Rabbit 684—the hemolytic strength remained unchanged for two days (Rabbits 674 and 643) or for three days (Rabbits 647, 642, and 644). This level marks the height of the response to the first injection. The effect of the second injection began, in some instances (Rabbits 674 and 642), to be apparent after three days, and in no animal was there any increase in the hemolysin content of the blood after the fifth day. The exceptional course of the curve in Rabbit 684, in which there was no pause in the increase of the rate of hemolysin production up to the final maximum, is due perhaps to two factors: first, the response to the second injection may have begun earlier than in the other rabbits; and secondly, the hemolysin content of the blood in this animal on the seventh day after the first injection was so low—1:40—that a relatively small increase could be easily detected.

The curve of hemolysin production as determined in this study differs in two respects from that of precipitin production as found by von Dungern. (a) In one instance, his rabbit "A," the height of the precipitin production was not reached until the eighth day after the first injection of the Majaplasma, and in the same animal the height of precipitin production following the second injection was not reached until the sixth day. (b) After reaching its height on the seventh or eighth day, the curve of precipitin production rapidly descends to a point at which it remains for a time stationary, whereas the curve of hemolysin production remains stationary from the day on which the height is reached.

The results of the preceding study may be applied as follows to the practical production in rabbits of a hemolytic serum against sheep corpuscles.

Two intravenous injections of 1, or at most 2 c.c. each of washed sheep corpuscles should be given at an interval of not less than four days to three rabbits. At the end of five days after the second injection, at least one of the rabbits will almost always yield a strongly hemolytic serum the hemolytic potency of which will be stable. If it is desired not to kill the animals, almost as much blood usually can be obtained by bleeding from the ear vein on two successive days (say

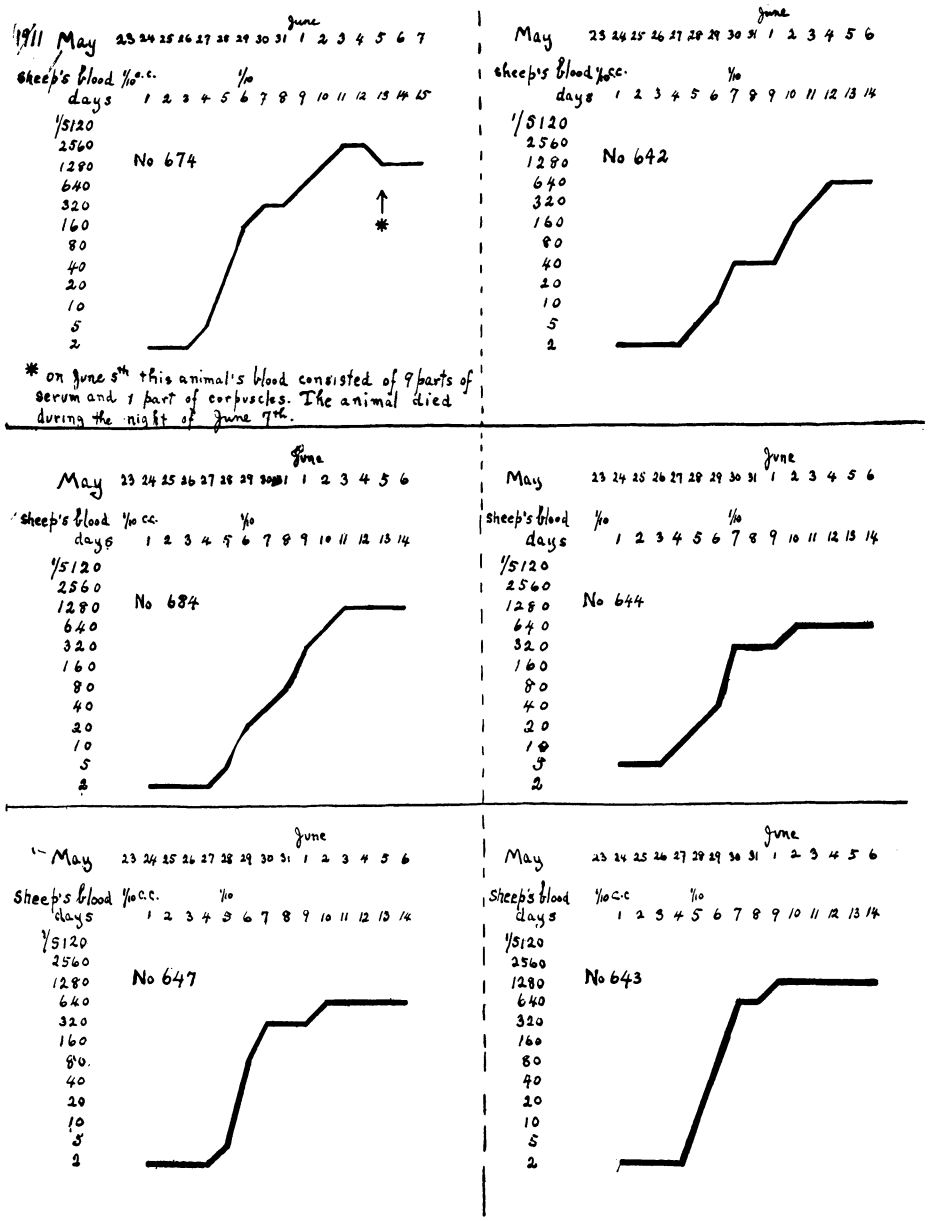


Chart 1.—The curve of hemolysin production in rabbits receiving two intravenous injections each of 0.1 c.c. of washed sheep corpuscles.

the sixth and seventh days after the second injection) as by bleeding to death. The potency of the serum obtained from the second bleeding is as high as that obtained from the first bleeding. The rabbits should not be used again for the production of amboceptor against sheep corpuscles.

In a single experiment in immunization with ox corpuscles, we have obtained results that are comparable with those obtained with sheep corpuscles.

Three normal rabbits received, at an interval of five days, two intravenous injections each of 1 c.c. of washed ox corpuscles. On the sixth day following the second injection, the hemolytic strength of the sera of the three rabbits was respectively 1:2500, 1:2500, and 1:320. After two months, during which the sera had been preserved in the ice-box with the addition of 0.25 percent of carbolic acid, the hemolytic strength of the sera was found to be unchanged.